Example 65

Assays for Metalloproteinase Activity

[1427] Metalloproteinases (EC 3.4.24.-) are peptide hydrolases which use metal ions, such as Zn^{2+}, as the catalytic mechanism. Metalloproteinase activity of polypeptides of the present invention can be assayed according to the following methods.

Proteolysis of alpha-2-macroglobulin

[1428] To confirm protease activity, purified polypeptides of the invention are mixed with the substrate alpha-2-macroglobulin (0.2 unit/ml; Boehringer Mannheim, Germany) in 1× assay buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, 25 μM $ZnCl_2$ and 0.05% Brij-35) and incubated at 37° C. for 1-5 days. Trypsin is used as positive control. Negative controls contain only alpha-2-macroglobulin in assay buffer. The samples are collected and boiled in SDS-PAGE sample buffer containing 5% 2-mercaptoethanol for 5-min, then loaded onto 8% SDS-polyacrylamide gel. After electrophoresis the proteins are visualized by silver staining. Proteolysis is evident by the appearance of lower molecular weight bands as compared to the negative control.

Inhibition of alpha-2-macroglobulin proteolysis by inhibitors of metalloproteinases

[1429] Known metalloproteinase inhibitors (metal chelators (EDTA, EGTA, AND $HgCl_2$), peptide metalloproteinase inhibitors (TIMP-1 and TIMP-2), and commercial small molecule MMP inhibitors) are used to characterize the proteolytic activity of polypeptides of the invention. The three synthetic MMP inhibitors used are: MMP inhibitor I, [IC_{50}=1.0 μM against MMP-1 and MMP-8; IC_{50}=30 μM against MMP-9; IC_{50}=150 μM against MMP-3]; MMP-3 (stromelysin-1) inhibitor I [IC_{50}=5 μM against MMP-3], and MMP-3 inhibitor II [K_i=130 nM against MMP-3]; inhibitors available through Calbiochem, catalog # 444250, 444218, and 444225, respectively). Briefly, different concentrations of the small molecule MMP inhibitors are mixed with purified polypeptides of the invention (50 μg/ml) in 22.9 μl of 1×HEPES buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, 25 μM $ZnCl_2$ and 0.05% Brij-35) and incubated at room temperature (24° C.) for 2-hr, then 7.1 μl of substrate alpha-2-macroglobulin (0.2 unit/ml) is added and incubated at 37° C. for 20-hr. The reactions are stopped by adding 4× sample buffer and boiled immediately for 5 minutes. After SDS-PAGE, the protein bands are visualized by silver stain.

Synthetic Fluorogenic Peptide Substrates Cleavage Assay

[1430] The substrate specificity for polypeptides of the invention with demonstrated metalloproteinase activity can be determined using synthetic fluorogenic peptide substrates (purchased from BACHEM Bioscience Inc). Test substrates include, M-1985, M-2225, M-2105, M-2110, and M-2255. The first four are MMP substrates and the last one is a substrate of tumor necrosis factor-α (TNF-α) converting enzyme (TACE). All the substrates are prepared in 1:1 dimethyl sulfoxide (DMSO) and water. The stock solutions are 50-500 μM. Fluorescent assays are performed by using a Perkin Elmer LS 50B luminescence spectrometer equipped with a constant temperature water bath. The excitation λ is 328 nm and the emission λ is 393 nm. Briefly, the assay is carried out by incubating 176 μl 1×HEPES buffer (0.2 M NaCl, 10 mM $CaCl_2$, 0.05% Brij-35 and 50 mM HEPES, pH 7.5) with 4 μl of substrate solution (50 μM) at 25° C. for 15 minutes, and then adding 20 μl of a purified polypeptide of the invention into the assay cuvett. The final concentration of substrate is 1 μM. Initial hydrolysis rates are monitored for 30-min.

Example 66

Characterization of the cDNA Contained in a Deposited Plasmid

[1431] The size of the cDNA insert contained in a deposited plasmid may be routinely determined using techniques known in the art, such as PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the cDNA sequence. For example, two primers of 17-30 nucleotides derived from each end of the cDNA (i.e., hybridizable to the absolute 5' nucleotide or the 3' nucleotide end of the sequence of SEQ ID NO:X, respectively) are synthesized and used to amplify the cDNA using the deposited cDNA plasmid as a template. The polymerase chain reaction is carried out under routine conditions, for instance, in 25 ul of reaction mixture with 0.5 ug of the above cDNA template. A convenient reaction mixture is 1.5-5 mM $MgCl_2$, 0.01% (w/v) gelatin, 20 uM each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer and 0.25 Unit of Taq polymerase. Thirty five cycles of PCR (denaturation at 94 degree C. for 1 min; annealing at 55 degree C. for 1 min; elongation at 72 degree C. for 1 min) are performed with a Perkin-Elmer Cetus automated thermal cycler. The amplified product is analyzed by agarose gel electrophoresis. The PCR product is verified to be the selected sequence by subcloning and sequencing the DNA product. It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

INCORPORATION BY REFERENCE

[1432] The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated herein by reference. In addition, the sequence listing submitted herewith is incorporated herein by reference in its entirety. The specification and sequence listing of each of the following U.S. and PCT applications are herein incorporated by reference in their entirety (filing dates shown in format "year-month-day" (yyyy-mm-dd)): Application No. 60/278,650 filed on 2001 Mar. 27, application Ser. No. 09/950,082 filed on 2001 Sep. 12, application Ser. No. 09/950,083 filed on 2001 Sep. 12, Application No. 60/306,171 filed on 19 Jul. 2001, application Ser. No. 09/833,245 filed on 2001 Apr. 12, Application No. PCT/US01/11988 filed on 2001 Apr. 12, Application No. 60/331,287 filed on 2001 Nov. 13, Application No. 60/277,340 filed on 2001 Mar. 21, Application No. PCT/US00/06043 filed on 2000 Mar. 9, Application No. PCT/US00/06012 filed on 2000 Mar. 9, Application No. PCT/US00/06058 filed on 2000 Mar. 9, Application No. PCT/